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ANALYSIS AND QUANTITATION OF THE CROSS PRESENTATION OF
TUMOR ANTIGENS USING THE HIV PROTEIN TRANSDUCTION
DOMAIN TRANSACTIVATING REGULATORY PROTEIN (TAT) TO
ALTER PRESENTATION

By

Jason Paul Aun

THESIS

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ABSTRACT

ANALYSIS AND QUANTITATION OF THE CROSS PRESENTATION OF TUMOR ANTIGENS USING THE HIV PROTEIN TRANSDUCTION DOMAIN TRANSACTIVATING REGULATORY PROTEIN (TAT) TO ALTER PRESENTATION

By

Jason Paul Aun

The hallmark of cross presentation is the immune system's ability to present an exogenously derived antigen in the class I major histocompatibility complex (MHC) thus stimulating CD8⁺ T_C cells. Exogenously derived proteins are normally presented in a MHC class II with the cells stimulating activation of CD4⁺ T_H cells which do not induce the desired cytotoxic effect. Inducing cross presentation is important in cancer immunotherapies because the tumor antigens are presented in the same fashion as exogenous proteins which do not provide the necessary cytotoxic effect. To stimulate a strong cytotoxic immune response, facilitating and optimizing cross presentation is paramount.

The purpose of this study was to analyze and quantitate the cross presentation of tumor antigens coupled with the protein transduction domain HIV-TAT. Protein transduction domains have the ability to enter a cell independent of any known receptor or endocytic activity. Coupling of a modified version of HER2 with HIV-TAT transferred HER2 to the cytoplasm of the antigen presenting cells. Labeling indicated that there was a slight increase in MHC class I expression using TAT-mHER2 compared to mHER2 alone. We conclude that the results of this study warrant further investigation into optimizing this promising technology.

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DEDICATION

This thesis is dedicated to my Sito and to my Aunt Julie.

Both lost their battles to cancer and it is my hope that in some small way this
research will help others fight on.

ACKNOWLEDGEMENTS

Completing this thesis would not have been possible without the help of several individuals. First, I would like to thank Dr. Winn for his support and knowledge in helping with my research and for working with my weightlifting training schedule every semester. I would also like to thank Drs. Becker and Rebers for taking time out of their busy schedules to be on my thesis committee. I owe tremendous gratitude to USA Weightlifting, the US Olympic Education Center, and NMU for giving me the chance to train and compete in a sport that I love and achieve my academic goals at the same time through the support of the B. J. Stupak Scholarship; this thesis would not have been possible without their help. There are several people that have assisted or instructed me and I am grateful for their help and input: Suzanne Dupler and Nathan Gazza for assisting me with the time consuming task of mHER2 production and Stephanie Humpula for helping me master the fluorescent microscope. As for the arduous task of producing TAT-mHER2, I have the love of my life and wife-to-be Jessica Fides to thank. Without her I would not have had any TAT-mHER2 for this study.

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And thank you so much to my family for all their support and love.

This thesis follows the format prescribed by *The Publication Manual of the American Psychological Association* and the Department of Biology.

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LIST OF ABBREVIATIONS AND ACRONYMS

° C – Degrees Celsius	21
µg – Microgram	19
µl – Microliter	20
µm – Micrometer	21
µM – Micromolar.....	25
Antp – Drosophila Antennapedia Homeotic Transcription Factor	12
APC – Antigen Presenting Cell	5
ATP – Adenosine Triphosphate.....	2
B Cell/Lymphocyte – Bone Marrow-Derived Cell/Lymphocyte (formerly Bursa of Fabricius-Derived Cell/Lymphocyte.....	5
BCA – Bicinchoninic Acid Assay	25
C – Carbon	3
C57BL/6 – Female Mouse 57 Black 6 (Denotes Original Breeding Stock).....	25
CD4 – Cluster of Differentiation Molecule 4	1
CD8 – Cluster of Differentiation Molecule 8	1
CD28 – Cluster of Differentiation Molecule 28	7
CD80 – Cluster of Differentiation Molecule 80	7
CDR2 – Complementarity Determining Region 2.....	11
CLIP – Class II Invariant Peptide	5

CO ₂ – Carbon Dioxide	26
C-terminus – Carboxyl-terminus	3
CTL – Cytotoxic T Lymphocyte.....	11
DAPI – 4',6-Diamidino-2-Phenylindole.....	29
DC – Dendritic Cell	5
DE3 – indicates that the host is a lysogen of λ DE3 and carries a chromosomal copy of the T7 RNA polymerase gene under control of the <i>lacUV5</i> promoter allowing for production of protein from target genes cloned in pET vectors by induction with IPTG.....	19
dH ₂ O – Distilled Water	22
DNA – Deoxyribonucleic Acid	12
EDTA – Ethylenediaminetetraacetic Acid.....	24
EGFR – Epidermal Growth Factor Receptor.....	13
ER – Endoplasmic Reticulum.....	4
erbB – Epidermal Growth Factor Receptor	13
Erp57 – Endoplasmic Reticulum Protein 57.....	4
FACS – Fluorescence-Activated Cell Sorting	37
FBS – Fetal Bovine Serum	27
FITC – Fluorescein Isothiocyanate Conjugate	28
FPLC – Fast Protein Liquid Chromatography	23
g – Standard Gravity	21

G ₁ – Gap 1 Phase	14
GuHCl – Guanidine Hydrochloride	20
H2-K ^b – Haplotype 2-Nonclassical Gene K	25
HC – Histocompatibility	9
HCl – Hydrochloric Acid	27
HCMV – Human Cytomegalovirus	8
HER – Human Epidermal Growth Factor Receptor	13
HER2EC1/2 – HER2 Extracellular	19
HER2IC1/4 – HER2 Intracellular	19
HIV-1 TAT – Human Immunodeficiency Virus-1 Transactivator of Transcription Protein	12
HLA-DM – Human Leukocyte Antigen-Nonclassical Class II MHC Molecule	6
HSP90 – Heat Shock Protein 90	13
HSV VP22 – Herpes Simplex Virus-1 DNA-Binding Protein Viral Protein 22	12
IFN- γ – Interferon- γ	2
IPTG – Isopropyl β -D-1-Thiogalactopyranoside	19
kDa – Kilodalton	25
LB – Luria-Bertani	19
LDS-PAGE – Lithium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	22
LMP2 – Low Molecular Mass Polypeptide 2	2

LMP7 – Low Molecular Mass Polypeptide 7	2
LMP10 – Low Molecular Mass Polypeptide 10	2
M – Molar	20
mAb – Monoclonal Antibody	15
MgCl ₂ – Magnesium Chloride	20
MHC – Major Histocompatibility Complex	1
mHER2 – Modified HER2 Construct Consisting of Extracellular HER2, SIINFEKL, and Intracellular HER2	18
MIIC – Class II MHC-Rich Endosomes	6
ml – Milliliter	15
mM – Millimolar	20
MOPS – 3-Morpholinopropane-1-Sulfonic Acid	22
NaCl – Sodium Chloride	20
NaOH – Sodium Hydroxide	27
<i>neu</i> – Name Derived from a Neuroglioblastoma Murine Cell Line	13
Ni ⁺⁺ – Nickel Ion	23
NiSO ₄ – Nickel Sulfate	23
nm – Nanometer	20
N-terminus – Amino-terminus	3
OD – Optical Density	20

OVA – Ovalbumin.....	11
P-Value – Probability Value	31
PBS – Phosphate Buffered Saline.....	24
PCD – Paraneoplastic Cerebellar Degeneration	11
pH – Potential of Hydrogen	5
<i>pLacI</i> – Promoter <i>lac</i> Inducibility (Repressor) Gene.....	19
PTD – Protein Transduction Domain	12
RBC – Red Blood Cell.....	26
RER – Rough Endoplasmic Reticulum.....	3
RNA – Ribonucleic Acid	20
Rnase A – Ribonuclease A	12
rpm – Revolutions per Minute	26
RPMI + 10% FBS – Roswell Park Memorial Institute Media with 10% Fetal Bovine Serum	25
S – Synthesis Phase.....	14
Sav-FITC – Streptavidin-Fluorescein Isothiocyanate Conjugate	28
SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	21
Sec61 – Secretory Pathway Protein 61	7
SIINFEKL – Serine-Isoleucine-Isoleucine-Asparagine-Phenylalanine-Glutamate- Lysine-Leucine	17
T Cell/Lymphocyte – Thymus-Derived Cell/Lymphocyte.....	1

TAP – Transporter Associated with Antigen Processing	4
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T _C Cell – T Cytotoxic Cell.....	1
T _H Cell – T Helper Cell	1
TKI – Tyrosine Kinase Inhibitor.....	15
US6 – Unspliced HCMV Glycoprotein 6	8
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β -gal – β -Galactosidase.....	12

INTRODUCTION

Major Histocompatibility Complexes and Antigen Presentation

The class I major histocompatibility complex (MHC) is present on almost all nucleated cells and derives antigen from endogenous proteins produced within the cell. Exceptions to this are immunologically privileged sites such as the anterior chamber of the eye, neurons in the brain, the cornea, testis, and the uterus (Goldsby, Kindt, & Osborne, 2000). Cells in these privileged sites express the tumor necrosis factor Fas ligand on their cell surface, which binds to CD8⁺ T_C cells expressing Fas receptor (Green & Ware, 1997). This binding causes the CD8⁺ T_C cells to undergo apoptosis thus avoiding tissue destruction by the immune system in these privileged sites (Green & Ware, 1997). Additionally, there is very little expression of MHC class I molecules in these sites, further inhibiting CD8⁺ T_C cell activity (Green & Ware, 1997). Cornea transplants take advantage of this immunologic privilege allowing corneas from donors to be transplanted without rejection by the immune system (Green & Ware, 1997). The CD8⁺ T_C cell is a specialized T lymphocyte that originates in the bone marrow and matures in the thymus. These cells are generally cytotoxic in nature and, in contrast to CD4⁺ T_H cells, kill targeted cells outright rather than strictly activating a further immune response (Goldsby *et al.*, 2000). Both T cell populations express distinctive membrane molecules known as cluster of differentiation molecules (CD8 and CD4) acting as signal transducers to elicit the appropriate reaction with their respective MHC molecules. T cytotoxic cells express CD8 that interacts with MHC class I molecules and T helper cells express CD4 that

interacts with MHC class II molecules. The MHC class I derives antigens endogenously through the cytosolic pathway and the MHC class II derives antigens exogenously through the endocytic pathway. To elicit a T cell immune response in either MHC class, extracellular antigens must first be internalized. After internalization of antigenic proteins through the plasma membrane by endocytosis from the extracellular fluid into the cytoplasm, internalized antigens are degraded by the proteasome (Ciechanover, 1994). The proteasome is a large, cylindrical, multifunctional protease complex with a central channel. This channel isolates the target protein for degradation, thereby preventing lysis of other proteins in the cytoplasm (Goldsby *et al.*, 2000). The proteins targeted for degradation by the proteasome often have the small protein ubiquitin attached to them (Ciechanover, 1998). Ubiquitin's primary function is to mark proteins for proteolysis through a complex cascade of enzymes (Ciechanover, 1998). Proteins marked for degradation are typically short-lived (internalized antigens), misfolded, or have a dissociation of subunits (Hershko & Ciechanover, 1998). Once conjugated to ubiquitin, the proteasome can degrade the marked protein by cleaving peptide bonds between two or three different amino acid combinations in an ATP-dependent process generating a variety of peptides terminating with hydrophobic or basic residues (Ciechanover, 1998). Two subunits of the proteasome, LMP2 and LMP7 are encoded within the MHC gene cluster and are induced by increased levels of the cytokine, interferon- γ (IFN- γ); another subunit, LMP10, is also induced by IFN- γ but it is not MHC encoded (Niedermann *et al.*, 1995). The peptidase activities of proteasomes containing LMP2, LMP7, and

LMP10 preferentially generate peptides with basic and/or hydrophobic residues that bind to MHC class I molecules (Niedermann *et al.*, 1995). These hydrophobic, and occasionally basic, residues that are part of the generated peptides act as anchor residues (at the carboxyl [C] terminal) to bind with the MHC class I molecule. All peptides examined to date that bind to MHC class I molecules contain a carboxyl-terminal anchor (Goldsby *et al.*, 2000). These peptides also possess an anchor residue at position two or positions two and three at the N terminal residue and at the C terminal residue pointing into the cleft which are also important for binding (Lankat-Buttgereit & Tampé, 2002). These antigenic peptides destined for interaction with the MHC class I molecule number only eight to 10 residues because the conformation of the peptide-binding cleft in MHC class I molecules is a closed groove (Goldsby *et al.*, 2000). Conversely, MHC class II molecules have an open peptide-binding cleft accommodating slightly longer peptides of 13-18 amino acids and do not require basic/hydrophobic residues for peptide binding (Goldsby *et al.*, 2000). These open and closed peptide-binding clefts arise from variations in the polymorphic residues, which are the amino acids that vary among different MHC alleles (Abbas & Lichtman, 2001). These residues are located in and around the cleft which is composed of paired α -helices resting upon a floor made up of an eight-stranded β -pleated sheet (Abbas & Lichtman, 2001).

The next challenge the cell must overcome is transporting the newly cleaved peptides to the rough endoplasmic reticulum (RER) where the MHC molecules are being synthesized by polyribosomes (Abbas & Lichtman, 2001).

This is accomplished by the transporter associated with antigen processing (TAP), a member of the ATP-binding cassette transporter family, which is a membrane spanning heterodimer consisting of TAP1 and TAP2 (Abbas & Lichtman, 2001; Solheim, Carreno, & Hansen, 1997). The TAP-transporter translocates peptides generated by the proteasome complex from the cytosol and actively pumps them into the lumen of the RER (Abbas & Lichtman, 2001; Lankat-Buttgereit & Tempé, 2002). Major histocompatibility complex class I loading includes not only TAP but the components tapasin, calreticulin, Erp57, MHC class I heavy chain and MHC class I- β_2 microglobulin (β_2m) dimers (MHC class I light chain) (Ackerman, Kyritsis, Tampé, & Cresswell, 2003; Guermonprez, *et al.*, 2003; Houde, *et al.*, 2003). The tapasin, calreticulin, and Erp57 function as chaperone proteins. Tapasin brings TAP into proximity with the MHC class I molecule and allows it to acquire an antigenic peptide (Ortmann *et al.*, 1997). Calreticulin is responsible for the folding and peptide-loading of newly synthesized molecules of the MHC class I protein (Culina, Lauvau, Gubler, & van Endert, 2004). The function of the resident ER thiol reductase Erp57 in MHC class I peptide assembly and loading is thought to contribute to the formation of disulfide bonds during the maturation of MHC class I chains (Lankat-Buttgereit & Tempé, 2002). Within the RER membrane, the newly synthesized MHC class I α chain associates with calnexin (a chaperone protein that assists in protein folding) until β_2m binds to the α chain (Goldsby *et al.*, 2000). The MHC class I α chain- β_2m heterodimer then binds to calreticulin and the TAP-associated protein tapasin (Solheim *et al.*, 1997). The physical association of the α chain- β_2m heterodimer with the TAP

protein promotes peptide capture by the class I molecule before the peptides are exposed to the luminal environment of the RER, where it has been demonstrated that unbound peptides are rapidly degraded (Goldsby *et al.*, 2000). As a result of binding, the MHC class I molecule becomes stable and can dissociate from the calreticulin and tapasin (Solheim *et al.*, 1997). The MHC class I α chain- β_2 -m heterodimer with its associated peptide fragment is transported from the RER to the plasma membrane by the Golgi apparatus, where it is expressed on the surface of the antigen-presenting cell (APC) and displayed to CD8⁺ T_C cells (Goldsby *et al.*, 2000).

The class II MHC is primarily presented on macrophages, dendritic cells (DCs), B lymphocytes, and endothelial cells which present antigens that have been internalized in phagocytic/endocytic vesicles (Abbas & Lichtman, 2001). Dendritic cells, which are professional antigen-presenting cells APCs, are the most important cells for initiating primary T cell responses and primarily present MHC class II (Abbas & Lichtman, 2001). After internalization into the endosome, the antigen is degraded into peptide fragments by proteolytic enzyme digestion by moving through three increasingly acidic compartments: early endosomes (pH 6.0–6.5); late endosomes or endolysosomes (pH 5.0–6.0); and lysosomes (4.5–5.0) (Goldsby *et al.*, 2000). The mechanism by which internalized antigen moves from one endocytic compartment to the next has not been conclusively demonstrated (Goldsby *et al.*, 2000). The fragments are attached to MHC class II molecules which prior to coupling with the antigen peptide was bound to a class II invariant peptide (CLIP) in the RER; this invariant

chain stabilizes the class II molecule before it has acquired the antigenic peptide (Goldsby *et al.*, 2000). To load the peptide onto the newly synthesized MHC class II molecule, the peptide exchange molecule HLA-DM facilitates the removal of CLIP from the binding cleft allowing peptides derived from the endocytosed protein to join in the endosomal compartment (possibly via the Golgi apparatus) (Abbas & Lichtman, 2001). The endosome is an intracellular membrane-bound vesicle into which extracellular proteins are internalized during antigen processing (Abbas & Lichtman, 2001). A subset of MHC class II-rich endosomes (MIIC) specialize in antigen processing and presentation by the class II MHC pathway (Abbas & Lichtman, 2001). The antigen/MHC class II molecule complex is then transported via the Golgi apparatus to the cell surface for expression on the APC and displayed to $CD4^+$ T_H cells (Abbas & Lichtman, 2001).

Cross Presentation of Antigens

There is increasing evidence of a phenomenon known as cross presentation; that is exogenous proteins internalized by phagocytosis or pinocytosis, are presented in MHC class I molecules to $CD8^+$ T cells (Saveanu & van Endert, 2005). Cross presentation is a mechanism by which professional APCs (typically DCs) display exogenous and self-antigens of another cell (such as a tumor cell) and activate (or prime) a naïve $CD8^+$ T_C cell (Abbas & Lichtman, 2001). This occurs, for example, when a cancerous cell is ingested by a professional APC, and the tumor antigens are processed and presented in association with the MHC class I molecules, as opposed to MHC class II (Abbas

& Lichtman, 2001). The professional APC also provides costimulation to fully activate the T cell which involves the interaction of two proteins, CD80 on the APC and CD28 on the T cell (Abbas & Lichtman, 2001). *In vivo*, DCs acquire endogenous antigens from infected cells in the periphery, and then migrate to the lymph nodes where they display antigenic peptides in association with MHC class I molecules (Groothuis & Neefjes, 2005). The mechanism by which cross presentation occurs is poorly understood but recent research shows that it occurs in early phagosomes prior to the formation of the phagolysosome (Saveanu & van Endert, 2005). The phagosome is an intracellular vacuole formed by the fusion of pseudopodia around a particle undergoing phagocytosis (Goldsby *et al.*, 2000). The phagosome fuses with a lysosome to form a phagolysosome (Abbas & Lichtman, 2001). Early macrophage phagosomes are formed mainly by ER membranes; these harbor Sec61 which has been shown to retrotransport ER proteins to the cytoplasm for degradation by the proteasome (Saveanu & van Endert, 2005; Gagnon, 2002). The early phagosomes contain all the functional elements for TAP-dependent loading of MHC class I molecules (Saveanu & van Endert, 2005). By completely separating the processing of exogenous antigens from that of endogenous ones, the existence of an autonomous ER-phagosome contributes to the efficiency of cross presentation of ER-mediated phagocytosed material (Saveanu & van Endert, 2005). The uptake of particulate phagocytosed antigens that eventually become cross presented is much more efficient than the uptake of soluble antigens through pinocytosis (Kovacsovics-Bankowski, Benacerraf, & Rock, 1993; Saveanu & van Endert, 2005). This was demonstrated

by Ackerman *et al.* (2003), by incubating DCs with a soluble peptide fragment of US6 from the human cytomegalovirus (HCMV). US6 is a HCMV-encoded type I glycoprotein peptide that inhibits peptide trafficking from the cytosol into the endoplasmic reticulum and subsequent peptide loading of MHC class I molecules (Kyritsis *et al.*, 2001). US6 inhibits peptide loading by inhibiting TAP function by binding to its luminal domain (Kyritsis *et al.*, 2001). The result of this inhibition was a reduction (up to 90%) of MHC class I molecules being displayed on the cell surface and inhibition of about 70% of the cellular TAP transporter pool leading to inhibition of endogenous antigen presentation (Kyritsis *et al.*, 2001). This shows that US6 had to gain access to the complete perinuclear ER (Kyritsis *et al.*, 2001). To corroborate this, Kyritsis *et al.* (2001) incubated β_2m -deficient DCs with exogenous β_2m and found that the exogenous β_2m localizes rapidly to the perinuclear ER, associates with MHC class I heavy chains bearing ER-typical immature glycans, reconstitutes cell surface class I expression, and normalizes presentation of endogenous antigen (Kyritsis *et al.*, 2001; Saveanu *et al.*, 2005). Both US6 and β_2m do not access the ER of macrophages, due to the rapid degradation of endocytosed soluble proteins in macrophages (Saveanu *et al.*, 2005). Limiting proteolytic activity in early endocytic vesicles is another requirement for efficient cross presentation—as demonstrated by macrophages, whose aggressive endocytic machinery focuses on pathogen destruction at the expense of cross presentation capacity (Saveanu *et al.*, 2005).

Traditionally, the CD8⁺ paradigm states that CD8⁺ T cells can only recognize endogenously synthesized antigens, whereas CD4⁺ T cells recognize

exogenous antigens (Larsson, Fonteneau, & Bhardwaj, 2001). This paradigm failed to take into account the observation that immunity develops spontaneously to viruses that cannot infect professional APCs (such as Epstein-Barr virus), or to tumors and allografts that by themselves are poor APCs (Larsson *et al.*, 2001). In 1976, Bevan showed that mice immunized with cells that express foreign minor histocompatibility (HC) antigens mounted an antigen-specific response that was restricted to self-class I MHC, demonstrating that exogenous pathways are important in the induction of CD8⁺ T cell responses (Larsson *et al.*, 2001). Minor HC antigens are proteins expressed outside of the MHC loci; these antigens are a contributing factor to graft rejection and are only recognized when they are presented in the context of self-MHC molecules unlike major histocompatibility antigens which are recognized directly by T_H and T_C cells, i.e. alloreactivity (Goldsby *et al.*, 2000). This study formed the basis of the postulate that antigens presented exogenously by donor cells to professional APCs were able to be presented on MHC class I molecules, i.e. cross presentation (Larsson *et al.*, 2001). Several features were found from these cross presentation studies: efficient cross presentation of antigen required bone-marrow-derived cells such as dendritic cells, diverse groups of antigens could access MHC class I by exogenous pathways, and in some cases, cross presentation was dependent upon the presence of TAP, suggesting that antigens have to access the cytoplasm of professional APCs to converge with conventional endogenous processing pathways (Larsson *et al.*, 2001).

It has been demonstrated that the components of the Sec61 complex, a translocon that forms a pore on the ER membrane, are also associated with phagosomes (Houde *et al.*, 2003). Sec61's primary function is the co-translational transfer of newly synthesized proteins into the RER (Lehner & Cresswell, 2004). It has been hypothesized that proteins internalized through endocytosis are transferred from components of the Sec61 complex (Houde *et al.*, 2003). After transferring from the endosome to the cytosol, the proteins would undergo ubiquitination and proteasomal degradation in the cytoplasm to generate the correct peptides for MHC class I loading (Houde *et al.*, 2003). These peptides would be transported into either the RER lumen through the TAP complex to form MHC class I-peptide complexes (Houde *et al.*, 2003) or be transported back into the same phagosome from which they originated (Ackerman, Kyritsis, Tampé, & Cresswell, 2005; Guermonprez *et al.*, 2003). The MHC class I-peptide complexes formed in the RER would be transported to the cell surface through the secretory pathway, while the MHC class I-peptide complexes formed in the phagosomes would be transported to the cell surface through the membrane recycling machinery of endocytic/phagocytic organelles (Houde *et al.*, 2003). After the antigen has been loaded onto the MHC class I molecule, it is then transported to the cell surface by the Golgi apparatus and then with an exocytic vesicle where it is finally expressed on the surface of the APC and displayed to CD8⁺ T_C cells.

Albert *et al.* (1998) demonstrated that human DCs could phagocytose apoptotic influenza-infected monocytes and stimulate resting CD8⁺ T cells to

develop into cytotoxic T lymphocytes (CTLs). Dendritic cells pulsed with apoptotic tumor cells also primed tumor specific CTLs (Larsson *et al.*, 2001). Additionally, studies showed that splenic DCs isolated after *in vivo* priming with ovalbumin (OVA)-loaded β_2m -deficient splenocytes presented OVA antigens to OVA-restricted MHC class I $CD8^+$ T cells (Larsson *et al.*, 2001). Cross presentation was TAP-dependent and restricted to the lymphoid $CD8^+$ DC subset, even though myeloid $CD8^-$ DCs acquire antigens *in vivo* (den Haan, Lehar, & Bevan, 2000); $CD8^-$ DCs lack the co-receptor T cell accessory molecule CD8 which is necessary for adhesion and signal transduction and thus are unable to cross present (Abbas & Lichtman, 2001; den Haan *et al.*, 2000).

Studies have also been done on patients with paraneoplastic cerebellar degeneration (PCD) who also had limited underlying cancer (usually breast or ovarian) (Albert *et al.*, 1998). These patients also had antibodies toward complementarity determining region 2 (CDR2) antigen, which is normally expressed in immune-privileged sites (Albert *et al.*, 1998). Dendritic cells from these patients phagocytosed apoptotic tumor lines that expressed CDR2 and induced potent anti-CDR2 cytotoxicity from autologous T cells (Albert *et al.*, 1998). These data suggest that, in PCD, cross presentation of tumor antigens by DCs provides the initial stimulus for CTLs *in vivo* (Larsson *et al.*, 2001). Several additional studies have confirmed that human DCs can cross present antigens from apoptotic tumor cells to $CD8^+$ T cells (Larsson *et al.*, 2001). As evident by these studies, cross presentation might be essential for the generation of tumor immunity.

Protein Transduction Domains

It has proven difficult to generate protective CD8⁺ T cell immunity to microbes, viruses, and cancer in humans with the current vaccine strategies employing peptides, plasmid DNA, subunit vaccines, and inactivated viruses (Larsson *et al.*, 2001). One possible reason is that delivery of exogenous antigens to DCs has not been optimized (Larsson *et al.*, 2001). Protein transduction domains (PTDs) have been described as short peptides that are able to penetrate the plasma membrane and this transducing property can be conferred upon other proteins when fused with the PTD (Ford, Souberbielle, Darling, & Farzaneh, 2001). Several PTDs have been described in recent years, the human immunodeficiency virus-1 transactivator of transcription protein (HIV-1 TAT) in 1998, Drosophila Antennapedia homeotic transcription factor (Antp) in 1991, and herpes-simplex-virus-1 DNA-binding protein viral protein 22 (HSV VP22) in 1997. Neither the mechanism of protein transduction nor the biological function, if any, of PTDs is understood. It has been demonstrated that transduction of PTDs does not occur through the normal receptor-, transporter-, endosome-, or absorptive-endocytosis-mediated processes (Schwarze, Hruska, & Dowdy, 2000). Treatment with drugs that inhibit cellular transport does not effect transduction (Del Gaizo Moore & Payne, 2004). It has been demonstrated that linkage of molecules thought to be unable to penetrate the plasma membrane to PTDs confers the ability of the nonpermanent molecule to cross the membrane. In 1994, Fawell and colleagues demonstrated that large molecules such as β -galactosidase (β -gal), horseradish peroxidase, Rnase A and domain III of

Pseudomonas exotoxin A, when chemically cross-linked with TAT peptides were carried into cells *in vitro*. Fawell *et al.* (1994) also demonstrated that β -gal activity was present *in vivo* in liver, kidney, and lung tissues as well as heart muscle fibers, the red pulp area of the spleen and even in the central nervous system indicating the TAT- β -gal fusion was able to cross the blood-brain barrier. One possible mechanism of entry proposed by Schwarze *et al.* (2000) involves direct penetration of the lipid bilayer caused by the localized positive charge of the PTD in which the momentum of the molecule drives the covalently attached ‘cargo’ into the cytoplasm (Figure 1). After transduction, the membrane energetics would then favor reformation of an intact lipid bilayer (Schwarze *et al.*, 2000). The composition of PTDs have a moderate to high number of the basic amino acids arginine and lysine, which might be important for contact with the negatively charged inner face of the lipid bilayer (Schwarze *et al.*, 2000). Schwarze’s group has suggested that denatured proteins may be transduced more efficiently than correctly folded proteins and then correctly refolded by chaperonins such as heat shock protein 90 (HSP90) also shown in Figure 1 (Ford *et al.*, 2001; Schwarze *et al.*, 2000).

HER2

The human epidermal growth factor receptor (EGFR) family of tyrosine kinases consists of EGFR (HER1, erbB1), HER2 (erbB2, HER2/*neu*), HER3 (erbB3) and HER4 (erbB4) (Slamon *et al.*, 1989). The human epidermal growth factor receptor HER2 was discovered in the early 1980s, when a mutationally activated form of its murine homolog *neu* was identified in a search for oncogenes

in a carcinogen-induced rat tumorigenesis model (Moasser, 2007; Shih, Pady, Murray, & Weinberg, 1981; Slamon *et al.*, 1989). Its human homologue, *HER2* was simultaneously cloned and found to be amplified in a breast cancer cell line (King, Kraus, & Aaronson 1985; Moasser, 2007). The transforming potential of *HER2* differs from that of *neu* in that *HER2* is tumorigenic through overexpression while *neu* requires mutational activation (Moasser, 2007). The relevance of *HER2* to human cancer was established when it was discovered that approximately 25–30% of breast cancers have amplification and overexpression of *HER2* and these cancers have worse biologic behavior and prognosis (Moasser, 2007, Slamon *et al.*, 1989). The expressed protein, *HER2*, is a transmembrane protein that spans the cell membrane seven times and is approximately 500 amino acids in length. Human epidermal growth factor receptor 2's normal function is as a cell membrane surface-bound receptor tyrosine kinase involved with signal transduction leading to cell differentiation and growth (Olayioye, 2001). Varied mechanisms for *HER2* involvement in growth and differentiation have been proposed; however, no unified model has gained wide acceptance (Moasser, 2007). Three mechanistic models that have been proposed involve abnormalities in signaling and all involve the overexpression of *HER2*, either in homodimeric form or heterodimeric form coupled with either *EGFR* or *HER3*. All three models point to a cascade effect of signals leading to any of the following: G_1/S cell cycle deregulation, loss of cellular polarity, dysregulation of cyclin D1 (a G_1/S regulator), tumor invasion, tumor proliferation, and an increase in tumor metabolism and survival (Moasser, 2007). There are typically 25 to 50 copies of

the *HER2* gene in normal cells (Moasser, 2007). In cancerous cells there can be a 40- to 100-fold increase in HER2 expression, resulting in up to two million receptors expressed on the tumor cell surface (Lohrisch & Piccart, 2001; Moasser, 2007; Venter, Tuzi, Kumar, & Gullick, 1987) illustrating the sheer number of aberrant signaling receptors.

Human epidermal growth factor receptor 2 is an attractive target for drug therapy because it is a cell membrane receptor-based protein allowing for antibody-based treatments to be utilized. Several HER2-targeting therapeutic strategies have been utilized, most notably the humanized monoclonal antibody (mAb) trastuzumab. Trastuzumab's exact role in inhibiting HER2 tumors has not been fully elucidated (Moasser, 2007). It is thought that trastuzumab acts on the extracellular segment of HER2 causing cellular arrest during the G₁ phase of the cell cycle thereby reducing growth of the tumor (Kute *et al.*, 2004). Treating patients with early-stage HER2 positive tumors with trastuzumab in conjunction with chemotherapy (after tumor resection) has shown significant prolonging of disease-free survival and reduction of disease recurrence (Moasser, 2007). However, 70% of patients do not respond to trastuzumab and resistance to the drug develops rapidly in almost every patient treated (Kute *et al.*, 2004). Another negative aspect of trastuzumab treatment is cost; the full course of treatment is approximately \$70,000 (Fleck, 2006).

Tyrosine kinase inhibitors (TKIs), such as imatinib, have shown promise as well for inhibiting HER2 positive tumor growth. Potentially, TKIs have a desired advantage over monoclonal antibodies such as trastuzumab (Moasser,

2007). While trastuzumab is only able to bind to the extracellular segment of HER2 (being an antibody, which are cell-impermeable), TKIs are cell-permeable and can potentially inhibit the ligand-dependent and -independent kinase activity of HER2 residing within the intracellular domain (Moasser, 2007). This strategy could be effective because kinase activity is essential for the oncogenic function of HER2 (Moasser, 2007). Imatinib has shown positive treatment results in chronic myeloid leukemia and gastrointestinal stromal tumors and these successes led to treatment of HER2-positive tumors as well (Arteaga, 2003). However, TKIs are not target-specific like antibodies and their off-target effects potentially limit their therapeutic value compared with antibodies (Moasser, 2007). The development of HER2-specific TKIs has shown mixed to mediocre results as well (Moasser, 2007).

Trastuzumab and TKIs are two examples of treatments that have, at best, moderate potential. Using PTDs is an attractive methodology because of their ability to penetrate virtually any cell, independent of a cell receptor. This gives rise to the potential of using PTDs to elicit an immune response by coupling an antigen to the PTD that can be processed by dendritic cells and then presented to T cells. This would potentially bypass any problems related to resistance, specificity, or toxicity.

Current Approach

The current study takes advantage of PTD technology that may allow the transport of a protein directly into the cytosol and cause the protein to be processed by the endogenous antigen pathway and be expressed preferentially in a

MHC class I molecule. For this study, TAT PTD was used. The transactivator of transcription protein has shown moderate promise in stimulation of T cells *in vitro* in previous studies conducted in our laboratory. TAT will be coupled with a modified version of a human epidermal growth factor receptor protein, HER2. The modified construct was produced (linked with PTD-TAT) by cloning into a bacterial vector and consists of four components: TAT, the extracellular component of HER2, the eight amino acid sequence SIINFELK from ovalbumin (OVA₂₅₇₋₂₆₄), and the intracellular component of HER2. Collectively, this construct is denoted as TAT-mHER2 (m denotes modified).

One of the approaches that has been widely used in cancer immunotherapy has been to use antigens found on tumor cells to stimulate a cytotoxic immune response. By isolating APCs and then exposing them to the specific antigen, either in its native or a modified form, the APCs on reinfusion into the patient may be able to stimulate a specific immune response directed at the tumor cells bearing those antigens. However, one of the keys to immunotherapy is the ability to present antigens in MHC class I molecules and stimulate CD8⁺ T cytotoxic cells since the majority of proteins presented in MHC class I molecules are cytoplasmic and newly synthesized proteins which are degraded by the proteasome. These peptides produced by the proteasome are transported into the ER by TAP. Peptides derived from exogenous antigens such as would be the case if APCs were pulsed with soluble antigen and internalized by endocytosis are thought to be displayed primarily by MHC class II molecules and subsequently stimulate CD4⁺ T helper cells. Our hypothesis is that using protein transduction

domains, short peptides that are able to penetrate the plasma membrane, may confer their transducing property on other proteins when expressed as fusions with the PTD. The advantage of this technology, over just pulsing APCs with soluble antigen, would be that transport of a protein antigen directly into the cytosol might allow the antigen to be processed by the endogenous antigen pathway and expressed preferentially in a MHC class I molecule.

Using mHER2 alone (soluble protein) and TAT-mHER2 (soluble protein fused to a PTD) we hypothesize that there will be a greater degree of MHC class I presentation as measured by immunofluorescence using a monoclonal antibody specific for presentation of our mHER2 in MHC class I molecules.

MATERIALS AND METHODS

TAT-mHER2 and mHER2 Production

A DNA sequence was designed for the TAT protein and the mHER2 component which were then linked. This sequence was ligated into a plasmid vector (pTriEX, Novagen) and then transformed to *Escherichia coli* cells (Figure 2). The vector (pTriEX) was first transformed into Novablue *E. coli* cells (Novagen) which produce high yields of plasmids. The plasmids were isolated using a plasmid isolation kit from Qiagen and then the plasmids were used to transfect a second *E. coli* strain, Rosetta (DE3) *pLacI* cells. These cells are engineered to express large quantities of repressor proteins from the *LacI* regulatory gene to allow for optimal cell growth. After induction with isopropyl β -D-1-thiogalactopyranoside (IPTG), which binds to the repressor proteins leading to inactivation, large amounts of the target protein are produced by the *lac* operon. The first gene inserted into the plasmid was TAT (with a linker) followed by extracellular HER2 (HER2EC1/2), SIINFEEKL (amino acid residues 257-264 of chicken ovalbumin), and intracellular HER2 (HER2IC1/4) (the mHER2 plasmid construct did not have the TAT sequence). Selection for transformed TAT-mHER2/mHER2 expressing cells was achieved by taking advantage of selective antibiotic resistance to carbenicillin and chloramphenicol. These antibiotics were added to the initial culturing medium (carbenicillin 50 μ g/ml, chloramphenicol 34 μ g/ml), Luria-Bertani (LB) agar, to select for cells containing the recombinant DNA. After culturing, a single colony was transferred to LB broth, also treated with carbenicillin and chloramphenicol (same concentrations),

to allow the cells to proliferate overnight. Expression of TAT-mHER2/mHER2 protein was achieved by activating transcription of the *lac* operon with 1 mM IPTG which was added when optimal levels of *E. coli* growth were reached. A spectrophotometer set to 600 nm absorbance was used to find the optical density (OD) of the broth. When the OD₆₀₀ reached an absorbance of 0.5 (equal to 5×10^5 colony forming units/ml), with sterile LB broth used as the standard, the IPTG was added and incubated overnight. The transcription of TAT-mHER2/mHER2 resulted in the formation of cytoplasmic inclusion bodies.

Isolation of TAT-mHER2 and mHER2 Inclusion Bodies

The inclusion bodies were isolated and then solubilized in denaturing conditions in 6 M guanidine hydrochloride (GuHCl). After sufficient levels of *E. coli* growth were reached in the LB broth centrifugation was used to pellet the *E. coli* cells and the broth was removed by aspiration. The resulting *E. coli* pellet was resuspended in a buffered solution of 50 mM Tris and 25% sucrose at a pH of 8.0 (buffer A). Buffer A was prepared from a stock buffer consisting of 20 mM Tris and 100 mM NaCl at a pH of 8.0; buffers B and C, discussed below, are made from this stock buffer as well. After resuspension in buffer A, the *E. coli* cells were then lysed with a buffered 8.0 pH lysing solution consisting of 1% deoxycholate, 1% Triton X-100 (a nonionic surfactant), 0.3 mg/ml Sigma lysozyme, and 1 mM Calbiochem Protease Inhibitor Cocktail Set III (buffer B). After mixing the *E. coli* cells in the suspension of buffers A and B for 15 minutes, a solution of 1 mM MgCl₂ and 0.02 µl/ml of benzonase endonuclease was added to the suspension to remove all DNA and RNA components and reduce viscosity.

The lysates were then centrifuged at 6,800 x g for 10 minutes at 4° C in a Sorvall RC-5B centrifuge using the SLA-3000 rotor to pellet the lysates; the supernatant (buffer A and buffer B) was removed by aspiration and discarded. The lysate pellet was resuspended in a buffered solution consisting of 0.5% Triton X-100 (buffer C), centrifuged again at 6,800 x g for 10 minutes at 4° C, and the supernatant was aspirated; this was repeated two more times. The pellet was then resuspended in stock buffer followed by centrifugation at 6,800 x g for 10 minutes at 4° C and then the supernatant aspirated again. The pellet was resuspended again in stock buffer, transferred to an Oak Ridge tube and centrifuged at 11,000 x g for 10 minutes at 4° C. The supernatant was aspirated and discarded leaving a pellet consisting of purified inclusion bodies. The purified pellet of inclusion bodies was resuspended in an 8.0 pH solubilization buffer consisting of 20 mM Tris, 6 M GuHCl, 0.5 M NaCl, 10 mM imidazole, and 5 mM β -mercaptoethanol. The inclusion bodies were placed on a rotary mixer for 55 minutes at room temperature to ensure pellet resuspension. The Oak Ridge tube containing the suspension was then centrifuged at 9,800 x g for 10 minutes at 4° C to pellet the non-protein components of the suspension. The remaining supernatant, consisting of the solubilized inclusion bodies (protein), was sterile vacuum filtered through a 0.22 μ m cellulose acetate filter to remove any additional particulates that did not pellet and potential bacterial contaminants.

Sample Quantification

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the presence of TAT-mHER2/mHER2 protein (Figure 3) using

Invitrogen Pre-Cast NuPAGE Novex 4-12% Bis-Tris Gels. One hundred microliters of the TAT-mHER2/mHER2 protein samples (from the inclusion body isolation above), both in volumes of approximately 15 ml after sterile vacuum filtration, were prepared for SDS-PAGE first by suspending in 100% ethanol to take the protein out of solution from the 6 M GuHCl. The sample was cooled at -20° C for 10 minutes prior to micro-centrifugation for eight minutes at 4° C to pellet the protein. After removal of the supernatant, 90% ethanol was added, vortexed briefly to resuspend the pellet, and then cooled again at -20° C for 5 minutes prior to micro-centrifugation at 16,000 x g for eight minutes at 4° C. The supernatant was removed, the pellet allowed to air-dry and then resuspended in distilled water (dH₂O), 500 mM Invitrogen dithiothreitol, and 4 X lithium dodecyl sulfate PAGE (LDS-PAGE) buffer (Invitrogen). Two molecular weight standards were used to verify that the electrophoresis had run properly and to find the appropriate weight as well as presence of the TAT-mHER2/mHER2 proteins. Invitrogen SeeBlue[®] Plus2 Pre-Stained Standard allowed visualization of weight ranges during electrophoresis and Invitrogen Mark12[™] Unstained Standard allowed close estimation of the TAT-mHER2/mHER2 molecular weight. The pre-cast gels were loaded with TAT-mHER2/mHER2 samples and both molecular weight markers and run completely submerged in an Invitrogen Novex Mini-Cell gel box with NuPAGE MOPS SDS Running Buffer (Invitrogen) and NuPAGE Antioxidant (Invitrogen) for 54 minutes at 200 volts. After completion of electrophoresis, the pre-cast gel was removed from the gel box, opened, rinsed with dH₂O, and placed into a staining tray to stain overnight in Invitrogen

SimplyBlue™ SafeStain on an agitator. After staining, the gel was de-stained overnight with dH₂O then prepared for permanent archiving and easy observation of results by placing the gel between two sheets of cellophane soaked in Invitrogen Gel-Dry Solution and allowed to dry over night using an Invitrogen DryEase Gel Drying System.

TAT-mHER2 and mHER2 Protein Purification

TAT-mHER2 and mHER2 protein purification was achieved by protein capture on a nickel ion (Ni⁺⁺) chelating column, through Pharmacia Fast Protein Liquid Chromatography (FPLC). The plasmid transfected to the *E. coli* genome carried a sequence to code for six histidine amino acids to act as a tag for chelating with nickel sulfate (NiSO₄). An GE Healthcare HiTrap™ 5 ml Chelating HP Column was loaded with 100 mM of NiSO₄ and then washed with solubilization buffer to remove any excess NiSO₄. Imidazole in the solubilization buffer (20 mM Tris, 6 M GuHCl, 0.5 M NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) was used to elute non-specific proteins (less than six histidine tags) off the column into 20 mM imidazole fractions. The 20 mM imidazole was the result of the solubilization buffer (10 mM imidazole) mixed with an elution buffer consisting of 20 mM Tris, 6 M GuHCl, 0.5 M NaCl, and 300 mM imidazole at pH of 8.0. Specific proteins (six histidine tags) were eluted off the column using elution buffer alone. Next, the protein solubilized in the solubilization buffer was loaded onto the column. The histidine tags present on the protein allowed chelation with the NiSO₄, forming a protein-Ni⁺⁺ complex bound to the column. These Ni⁺⁺-bound proteins were eluted off the FPLC

column with imidazole. The non-specific proteins were eluted off the column first in 20 mM imidazole fractions collected in 10 ml increments. Determining when to collect the appropriate 20 mM imidazole fraction was achieved when the UV spectrophotometer on the FPLC showed a constant, mid-range plateau as the elution buffer moved through the column. When the UV spectrometer showed a significant increase in absorbance, 1 ml fractions were collected which consisted of 300 mM imidazole and these fractions consisted of the specifically-bound target protein (six histidine tags). Fraction collection was stopped when the UV peaks began to decline. All the 1 ml fractions were combined and 0.5 M ethylenediaminetetraacetic acid (EDTA) was added.

The purified TAT-mHER2/mHER2 protein was resuspended in 20 mM Tris, 6 M GuHCl, 0.5 M NaCl, 2 mM EDTA and was injected into a Pierce Slide-A-Lyzer dialysis cassette then dialyzed against a refolding/renaturation buffer consisting of 100 mM Tris, 0.5 M arginine, 2 mM EDTA, 2.5 mM reduced glutathione, and 0.5 mM oxidized glutathione at a pH of 8.0 to remove the GuHCl and to refold and renature the protein. This was done for 12 hours at 4° C on a stir plate. After 12 hours, the buffer was replaced with fresh refolding/renaturation buffer and dialyzed for another 12 hours at 4° C on a stir plate. The refolding/renaturation buffer was then replaced with phosphate buffered saline (PBS) and dialyzed again for 12 hours and repeated once more with fresh PBS for another 12 hours, both at 4° C on a stir plate. The refolded and renatured protein, now suspended in PBS, was suitable for *in vitro* use. Lastly, the protein was concentrated through ultrafiltration using an Amicon

Ultra-15 Centrifugal Filter Device (10,000 kDa nominal molecular weight limit).

The concentration of TAT-mHER2/mHER2 protein was determined by bicinchoninic acid assay (BCA). A Molecular Devices SpectraMax Plus Spectrophotometer (absorbance at 570 nm) operated by SOFTmax Pro 3.1.1 software was used to read the BCA assay absorbance results.

Antigen Presentation

Delivery of the TAT-mHER2/mHER2 was achieved by introducing the antigen to APCs cultured *in vitro* from murine (*Mus musculus*) spleen. Five different antigen concentrations were used, 2.5 μ M, 1.25 μ M, 0.625 μ M, 0.3125 μ M, and 0.15625 μ M, as well as a PBS control. Female H2-K^b positive C57BL/6 mouse spleens were used for the procedure. Spleen procurement began with cervical dislocation of the mouse followed by wetting of the mouse with 70% ethanol. To remove the spleen the skin was first lifted and cut with scissors to expose the abdominal muscle which was cut away as well to expose the spleen (appears dark-red in color and is long and thin in shape). The spleen was excised from the mouse and had all the connective tissue removed. Next, the spleen was placed into a Petri plate with Roswell Park Memorial Institute media with 10 ml of 10% fetal bovine serum (RPMI + 10% FBS). Using the frosted ends of two glass microscope slides, cleaned with 70% ethanol and rinsed with PBS, the mouse spleen was ground into a thin pulp into the RPMI + 10% FBS. The ground spleen and the RPMI + 10% FBS were then aspirated into a 15 ml centrifuge tube. An additional 5 ml of RPMI + 10% FBS was added to the Petri dish and rinsed on the slides to collect any remaining splenocytes; this was also added to the 15 ml

centrifuge tube. The splenocytes were centrifuged at 1250 rpm for 5 minutes to pellet the cells. The RPMI + 10% FBS was aspirated and 1 ml Sigma red blood cell (RBC) lysing buffer was added to lyse any RBCs. After one minute, 14 ml of RPMI + 10% FBS was added and the splenocytes were centrifuged again at 1250 rpm for 5 minutes to pellet the cells. Following centrifugation, the RPMI + 10% FBS was aspirated and 7 ml of fresh RPMI + 10% FBS was added to resuspend the splenocytes. The 7 ml RPMI + 10% FBS/splenocyte suspension was transferred to a 70 ml, 25 cm² cell culture flask and incubated for two to four hours at 37° C, 5% CO₂. After incubating, all RPMI + 10% FBS media and non-adherent cells were removed by aspiration. The flask was scraped to harvest the adherent cells (antigen presenting cells) and 12 ml of fresh RPMI + 10% FBS was added to resuspend the dendritic cells for aliquoting. A 24-well plate was prepared with 12 round coverslips (sterilized with 70% ethanol and rinsed with PBS) placed inside 12 wells where 1 ml of the RPMI + 10% FBS/dendritic cell suspension was aliquoted into each well and allowed to incubate for two hours at 37° C, 5% CO₂.

Immunolocalization of HER2 Presentation in MHC Class I

To determine the MHC class I expression levels of the TAT-mHER2 antigen, a modified version of a direct enzyme-linked immunoabsorbent assay to determine immunolocalization was employed and an Olympus BX51 Fluorescent Microscope was used to visualize the fluorescence of the labeled antigen. The antigen presenting cells obtained from the spleens of female H2-K^b positive C57BL/6 mice were grown on untreated glass cover slips placed inside 24-well

plates. After incubating for two hours in RPMI + 10% FBS media, the non-adherent cells were aspirated and 1 ml new media was added along with TAT-mHER2 acting as the antigen for the splenocyte APCs to uptake. Additionally, mHER2 (not cross-linked with TAT) were also tested serving as a comparative antigen in separate wells. Both antigens were added to each successive well in 25 μ l volumes of decreasing concentrations (diluted in PBS) with each successive dilution being half the concentration of the previous dilution, i.e. 2.5 μ M, 1.25 μ M, 0.625 μ M, 0.3125 μ M, and 0.15625 μ M, as well as a PBS control. After the addition of the antigen, the cells were incubated overnight to allow for antigen processing. Next, the media was aspirated and the cells fixed with 1 ml of 4% paraformaldehyde for 10 minutes, aspirated, and rinsed twice with PBS. The 4% paraformaldehyde solution was prepared immediately prior to use by adding 0.6 grams of paraformaldehyde, 12 ml of dH₂O, and 5 ml of 1 M NaOH to a beaker which was heated to 70°C and stirred until the paraformaldehyde solubilized (Doyle, 1996). The solution was cooled to 25°C, the volume was adjusted to 13.5 ml with dH₂O, and 1.5 ml of 10 X PBS was added to bring the final volume to 15 ml (Doyle, 1996). The pH was adjusted to 7.4 using drops of 10% HCl and the solution was sterile vacuum filtered through a 0.22 μ m cellulose acetate filter (Doyle, 1996). Twenty five microliters of goat serum were then added as a blocking agent, allowed to incubate for five minutes at 37° C, 5% CO₂, aspirated, and washed twice with BD Pharmingen Stain Buffer with 2% FBS (Dulbecco's Phosphate-Buffered Saline with 2% fetal bovine serum, 0.09% sodium azide, pH 7.4); this blocking step was repeated three times. After blocking, 25 μ l of the

primary antibody 25-D1.16, diluted to 1:100 in BD Pharmingen Stain Buffer with 2% FBS, was added to each well and incubated for 60 minutes at 37° C, 5% CO₂ and then washed three times over five minutes with PharMingen Stain Buffer.

This murine monoclonal antibody was produced by a hybridoma cell line provided by Angel Porgador and Jonathan Yewdell and it recognizes MHC class I molecules complexed with the amino acid sequence SIINFEKL (OVA₂₅₇₋₂₆₄) which TAT-mHER2 and mHER2 possess (Figure 4). The 25-D1.16 hybridoma cell line was originally cultured in Dulbecco's Modified Eagle Medium with 10% heat inactivated fetal calf serum and then Iscove's Modified Dulbecco's Medium with 10% FBS for antibody production. This hybridoma cell line produced copious amounts of 25-D1.16 antibody.

A secondary antibody, AbCam biotin-conjugated rat anti-mouse immunoglobulin, λ 2 and λ 3 light chain mAb (2B6ab) was then added to bind with the 25-D1.16 primary antibody (also diluted in Pharmingen Stain Buffer, but at double the concentration, 1:50) and allowed to incubate for an additional 60 minutes at 37° C, 5% CO₂. After washing three times over five minutes using PharMingen Stain Buffer, 25 μ l of PharMingen Streptavidin-Fluorescein Isothiocyanate Conjugate (Sav-FITC or simply FITC) was added in a concentration that was double the concentration of the secondary antibody, 1:25, to conjugate to the biotin complexed with the secondary antibodies for immunofluorescence. This step and all subsequent steps were performed in low-light conditions to preserve fluorescence. The cover slips within the wells were then mounted on slides and counter stained with Vector Vectashield Mounting

Medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) prior to analysis with the fluorescent microscope. Splenocytes expressing TAT-mHER2 or mHER2 displayed green fluorescent points on the cell surface (Figure 5) under the FITC filter and non-expressing cells were invisible or faintly colored. Three images were taken of each cell (using Applied Imaging fluorescent imaging software) under both the DAPI and FITC filters as well as an overlay of both pictures combined (Figures 5, 6, and 7). It is interesting to note that the concentration of TAT-mHER2 in these figures is at 0.15625 μ M. Diluting each antigen was done to determine when each reached a point of no longer being recognized by the primary antibody 25-D1.16. TAT-mHER2 and mHER2 were diluted as such: 2.5 μ M, 1.25 μ M, 0.625 μ M, 0.3125 μ M, 0.15625 μ M, and a PBS control.

RESULTS

To compare the expression level of both antigens (mHER2 and TAT-mHER2), cells counts were taken of the number of positive cells, cells showing FITC signaling, and the number of positive signals in each cell. The number of positive signals per cell was determined by counting individual FITC signals on a positive cell; for example, Figure 6 depicts nine positive FITC signals on that particular cell. The number of positive signals in each cell was divided by the number of positive cells resulting in the average number of positives per cell. These counts were taken at each concentration, including the mHER2 and TAT-mHER2 PBS controls. Only cells displaying the processed antigen in MHC class I were detectable in this assay due to the specificity of the 25-D1.16 mAb. The eight amino acid SIINFEKL sequence inserted between the intracellular and extracellular HER2 components must undergo antigen processing by the APCs in order to be recognized by the 25-D1.16 mAb. Any antigen that did not undergo cellular uptake and processing but was adhering APC's outer-membrane would not elicit any antigen/mAb interaction.

At every concentration level, except 0.15625 μ M, there were more positive TAT-mHER2 cells than mHER2 cells. Comparison of paired groups showed a significant difference for TAT-mHER2-treated cells at 1.25 μ M (Figure 8). The number of positive signals from the TAT-mHER2-treated cells was also higher than the mHER2-treated cells at every concentration level however the differences were not significant except at 1.25 μ M and 0.625 μ M (Figures 8 and 9). An asterisk denotes the statistically significant 1.25 μ M and 0.625 μ M TAT-

mHER2 concentrations in Figures 8 and 9. The PBS controls (which were not treated with either TAT-mHER2 or mHER2) also showed some low level positive signals, but these were attributed to nonspecific background staining (Figures 8 and 9). Interestingly, the average number of positives per cell was higher for mHER2 at all concentrations except at 0.625 μ M and 0.3125 μ M indicating that while less cells displayed mHER2, those that did displayed it at higher levels (Figure 10). These two concentration levels could show a possible optimal concentration level for TAT-mHER2 cell delivery.

A t-test for the two correlated samples (mHER2 versus TAT-mHER2) at each concentration was performed on both the number of positive cells and positives per cell to determine if the sum of the change between the two groups differed significantly from zero. The t-tests for these data revealed p-values that were insignificant at every concentration except at 1.25 μ M (positive cells) and 0.625 μ M (positives per cell):

Concentration	p-value (Positive Cells)
2.5 μ M	0.16647
1.25 μ M	0.00097
0.625 μ M	0.10891
0.3125 μ M	0.72527
0.15625 μ M	0.31934
PBS Controls	0.34791

Concentration	p-value (Positives per Cell)
2.5 μ M	0.56511
1.25 μ M	0.07529
0.625 μ M	0.00717
0.3125 μ M	0.72930
0.15625 μ M	0.55242
PBS Controls	0.33247

Additionally, three other concentration levels showed p-values that approached a p-value of 0.05: 2.5 μM and 0.625 μM for positive cells and 1.25 μM for positives per cell. Both the 1.25 μM and 0.625 μM concentrations showed a correlation between the positive cells and positives per cell counts.

DISCUSSION

Eliciting a cytotoxic immune response to cancer antigens, which are essentially self-antigens, has traditionally been a major obstacle, due to the APCs presenting these antigens in an MHC class II. The subsequent activation of CD4⁺ T_H cells typically leads to the release of cytokines which in turn activate B lymphocytes, macrophages, and CD8⁺ T_C cells. However, the CD8⁺ T_C cells cannot induce apoptosis due to the lack of costimulatory signaling from the target cell which leads to clonal anergy (Goldsby *et al.*, 2000). This signal is triggered by the interaction of CD80 on the APC with CD28 present on either CD4⁺ T_H or CD8⁺ T_C cells.

Another factor involved with this ineffective immune response is down-regulation of MHC class I expression on tumor cells which may allow the tumor to escape CTL-mediated recognition (Goldsby *et al.*, 2000). The immune response may play a role in selecting tumor cells expressing lower levels of MHC class I molecules by preferentially eliminating those cells expressing high levels of MHC class I molecules and, with time, malignant tumor cells may express progressively fewer MHC molecules and thus escape CTL-mediated destruction (Goldsby *et al.*, 2000). To compound this phenomenon, most tumors grow rapidly and it quickly becomes futile for the immune system to target and kill every tumor cell. Additionally, tumors can evolve to evade immune responses either by failing to express a tumor antigen on its MHC molecule, having an MHC mutation prevent MHC expression, or through production of immunosuppressive cytokines (Abbas & Lichtman, 2001).

Cross presentation is the immune system's response to these tumor evasion mechanisms. When an APC ingests a tumor cell, cross presentation occasionally occurs leading to a CD8⁺ T_C cell interaction, CD8⁺ T_C cell differentiation into a CTL, and finally apoptosis of tumor cells. The intracellular mechanisms of cross-presentation are still unclear, but seem to involve specialized subcellular compartments bearing characteristics of both the endoplasmic reticulum and the endosome (Guermonprez *et al.*, 2003). Taking advantage of this ability possessed by APCs allows the immune system to keep certain viruses and tumors in check even though these antigens would normally be presented in an endogenous manner. Increasing cross presentation in APCs and increasing the number of cross presenting APCs in general through the use of PTDs has been attempted by a number of researchers. The attractive feature of PTDs is the ability to enter a cell independent of any known receptor or endocytic activity. Add to this that PTDs can be coupled to other, much larger molecules and not lose their transducing properties presents a potential boon to immunotherapy research.

Kim *et al.* (1997) and Shibagaki and Udey (2002) were able to demonstrate the abilities of HIV-TAT PTD to induce the MHC class I pathway and thus showing an alternative to traditional methods of DC pulsing. Recent research by Tanaka, Dowdy, Linehan, Eberlein, and Goedegebuure (2003) and Viehl *et al.* (2005) demonstrated successful protein transduction of cancer antigens coupled with the HIV-TAT PTD and a subsequent induction of antigen-specific CTLs. All four studies demonstrated the efficiency by which proteins

could be introduced into the MHC class I pathway over the traditional use of tumor cell preparations such as tumor lysates and apoptotic tumor cells or DC-tumor cell fusions (Tanaka *et al.*, 2003). Other methods involving DNA, RNA, or viral vectors containing tumor antigen genes were also not as efficient as PTD transfer (Tanaka *et al.*, 2003).

Vaccine production has continually been stymied because of MHC class II elicitation in lieu of MHC class I. In all likelihood inducing just a MHC class I response would be virtually impossible, however Viehl *et al.* (2005) demonstrated that TAT coupled with mammaglobin (a breast cancer-associated protein) transduced dendritic cells induces both CD4 and CD8 mammaglobin-specific T cells. This scenario shows great promise that a vaccine could be developed using this technology. Effective antitumor immune responses are thought to require the contribution of both CD4⁺ and CD8⁺ T cells, making protein transduction of tumor antigens a viable strategy because of HIV-TAT's ability to cross the cell membrane of most mammals (Schwarze *et al.*, 2000; Viehl *et al.*, 2005).

The objective for this study was to analyze and quantitate the cross presentation of HER2 antigens coupled with the PTD HIV-TAT compared to HER2 alone. Our approach entailed using HIV-TAT to transport exogenous proteins to the cytosol where they would enter the endogenous pathway. Immunolocalization analysis was conducted using the mAb 25-D1.16. This mAb is specific for the peptide sequence SIINFEKL, the eight amino acid residues of chicken ovalbumin (residues 257-264), found between the extracellular HER2 and intracellular HER2 components of our TAT-mHER2 and mHER2 proteins and

labeling with mAb 25-D1.16 was used to display in an MHC class I molecule on murine splenic APCs. Labeling indicated that there was an increase in MHC class I expression using TAT-mHER2 compared to mHER2 and that increase was significant using 1.25 μ M and 0.625 μ M antigen concentrations (Figures 8 and 9). We expected to see greater MHC class I presentation following incubation with TAT-mHER2; while greater presentation was seen, the increased presentation was not as robust as expected.

While our hypothesis was not fully supported by the results of this study, similar studies conducted previously by Tanaka *et al.* (2003) and Viehl *et al.* (2005) showed more favorable results thus warranting further investigation. Several factors may have contributed to our results not supporting our hypothesis: the particular TAT-mHER2 sample used in this study may not have been fully functional; if the TAT-mHER2 was functional, there may have been no ubiquitination of the protein and therefore no antigen processing through the endocytic pathway; preparing the splenocytes for *in vitro* analysis may have placed too much stress on the murine splenic dendritic cells resulting in a decreased ability to process antigens; the assay developed for this study may not have been sensitive enough. Additionally, determining if the antigen was delivered into the cell or if the antigen just merely coated the cell surface, with no uptake, requires further study. Further, whether or not the antigen induced an immune response and if the actual cellular uptake of the antigen was through protein transduction need evaluation. Analyzing and quantitation of the cross presentation of HER2 tumor antigens could be optimized through the use of

fluorescence-activated cell sorting (FACS) or green fluorescent protein. Using both of these methods would allow the assay of live cells. Improving antigen delivery could potentially be improved through adding another PTD protein to the antigen (i.e. TAT-TAT-mHER2 or TAT-mHER2-TAT). Accomplishing this construct may require the use of cosmids, bacterial artificial chromosomes, or yeast artificial chromosomes to accommodate the larger genetic sequence. Additionally, determining if there is any specificity to PTD/cargo protein combinations might allow future study into optimizing antigen uptake. Expanding this study to an *in vivo* model may also improve more efficient TAT-mHER2 delivery and antigen presentation.

Continuing and expanding research into PTD biotechnology, particularly in regards to immunotherapy and the mechanistic means by which PTDs gain cell entry, could produce great gains in our ability to present cancer associated antigens to the immune system and may eventually lead to improved cancer patient outcomes.

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APPENDIX A

FIGURES

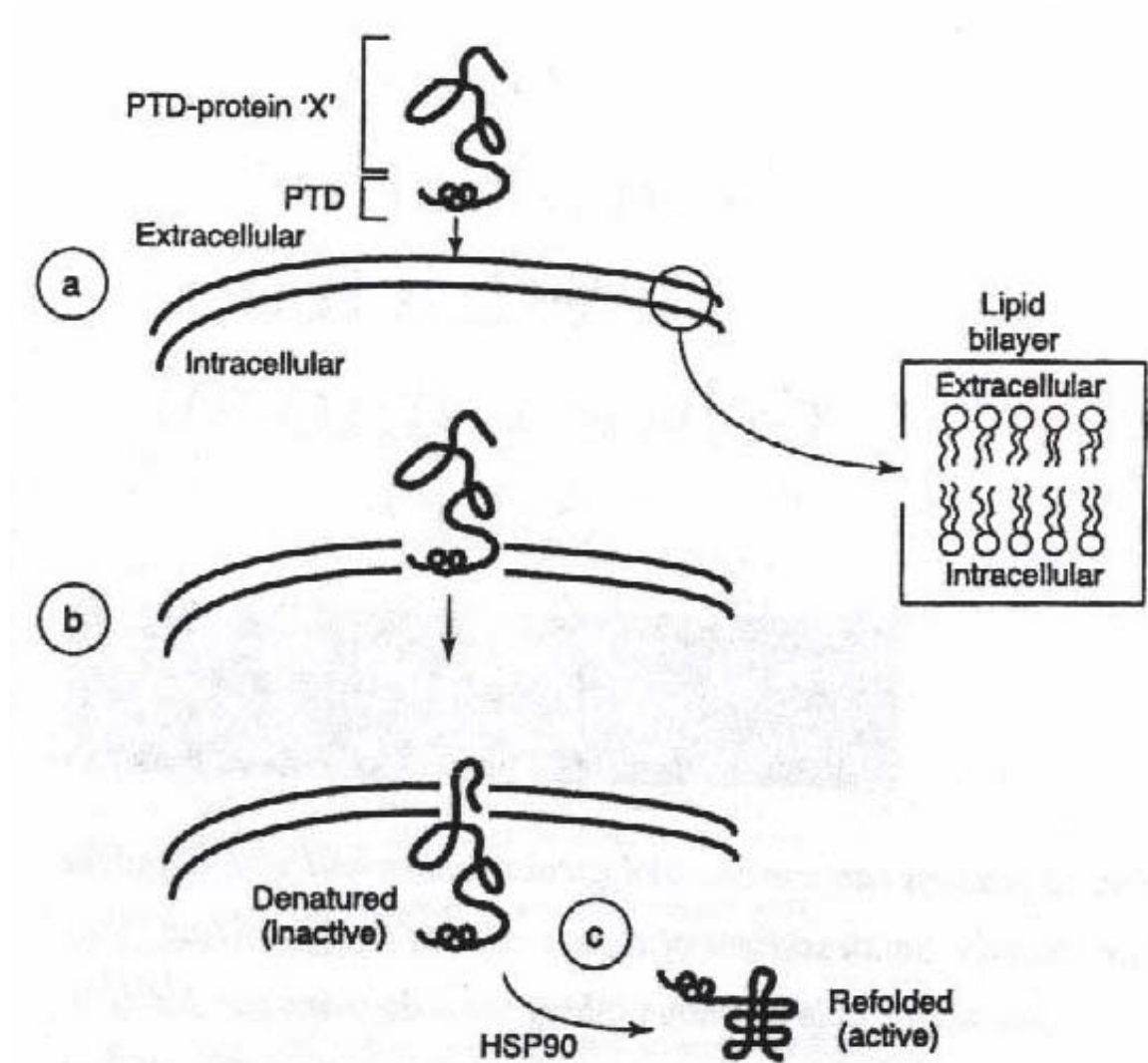


Figure 1: (a) PTD and protein 'X' complex, (b) protein 'X' being transported through the lipid bilayer by the PTD, and (c) refolding by HSP90 for reactivation (Schwarze *et al.*, 2000).

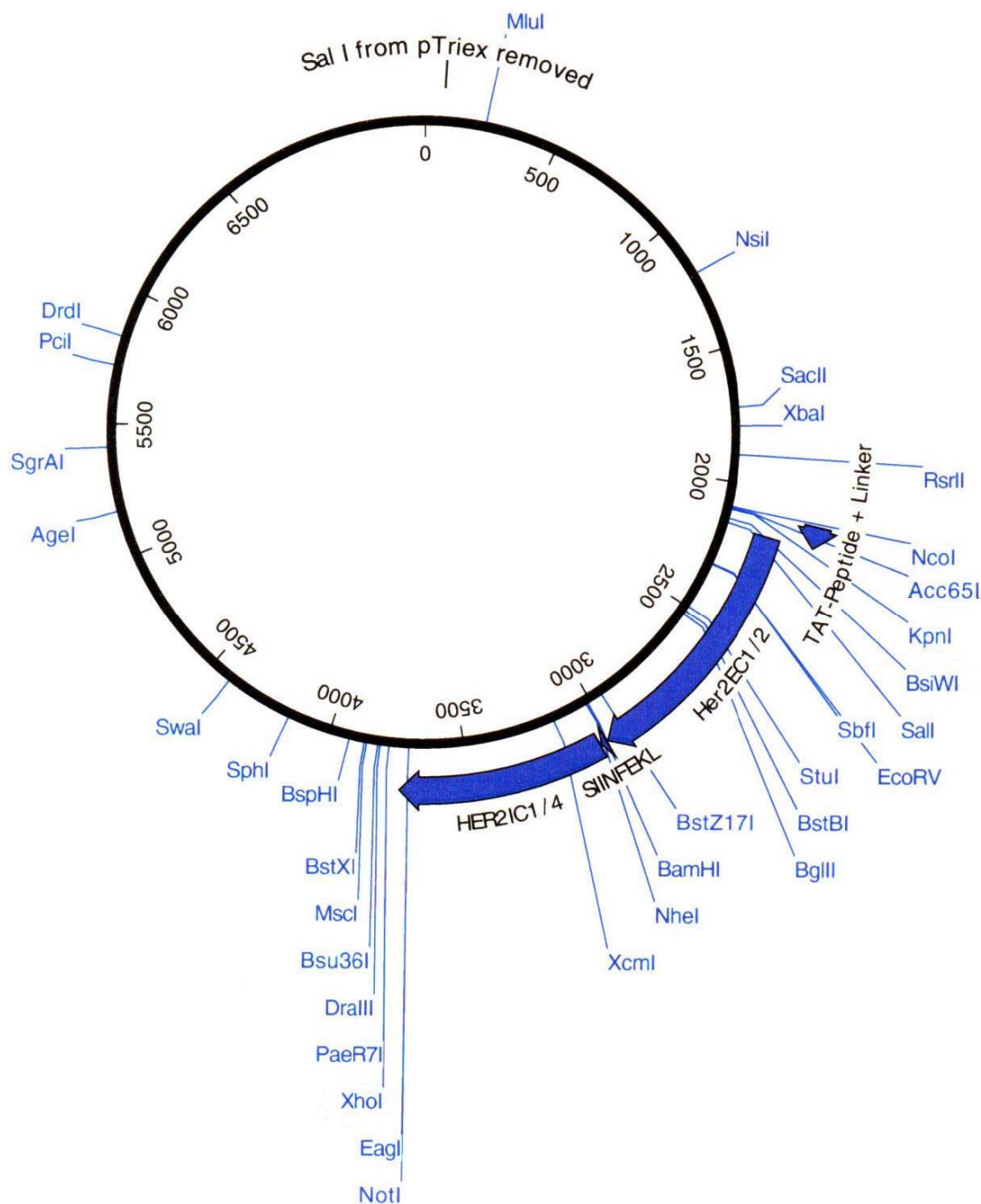


Figure 2: Plasmid pTriExSN-TAT-mHER2 containing PTD-TAT, extracellular and intracellular HER, and SIINFEKL. The gene encoding TAT was inserted first with a linker followed by extracellular HER2 (HER2EC1/2), SIINFEKL, and intracellular HER2 (HER2IC1/4).

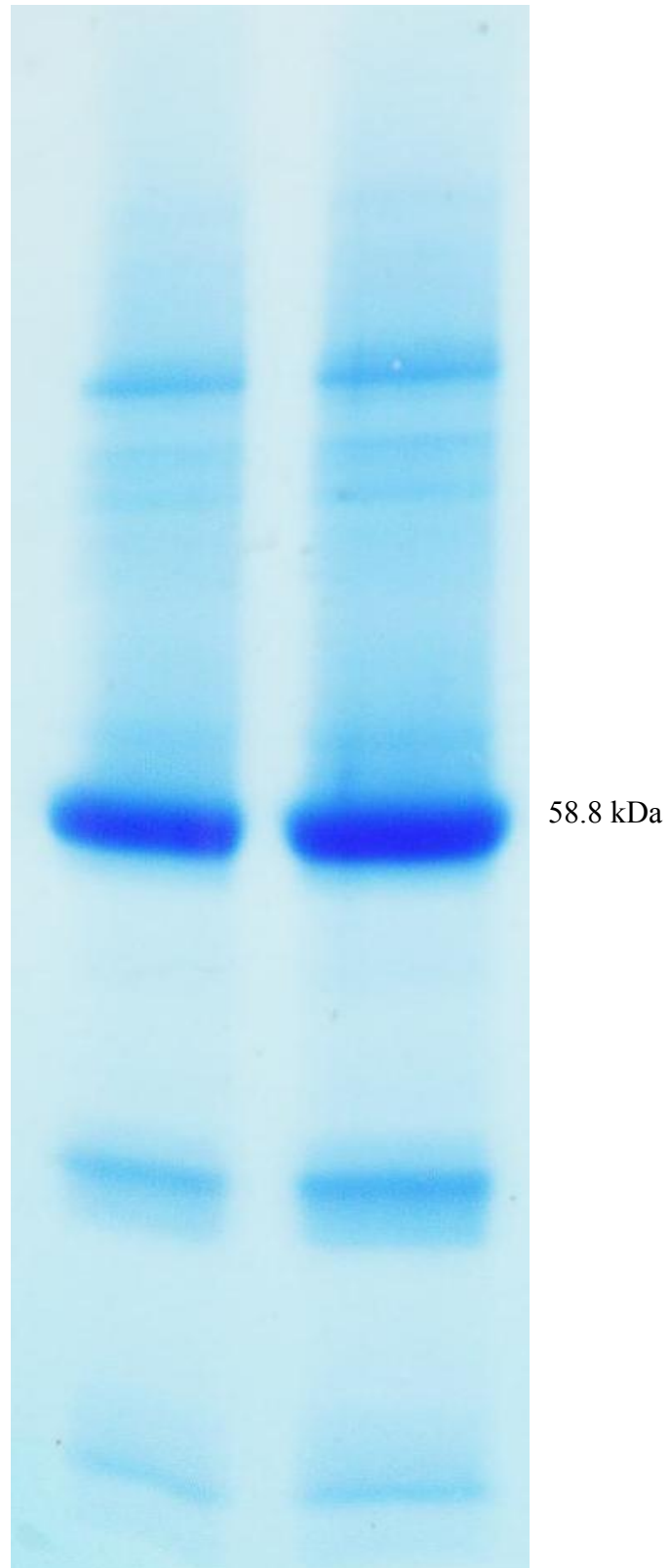


Figure 3: SDS-PAGE confirming presence of TAT-mHER2. The left lane contains 10 µl of sample; the right lane contains 15 µl.

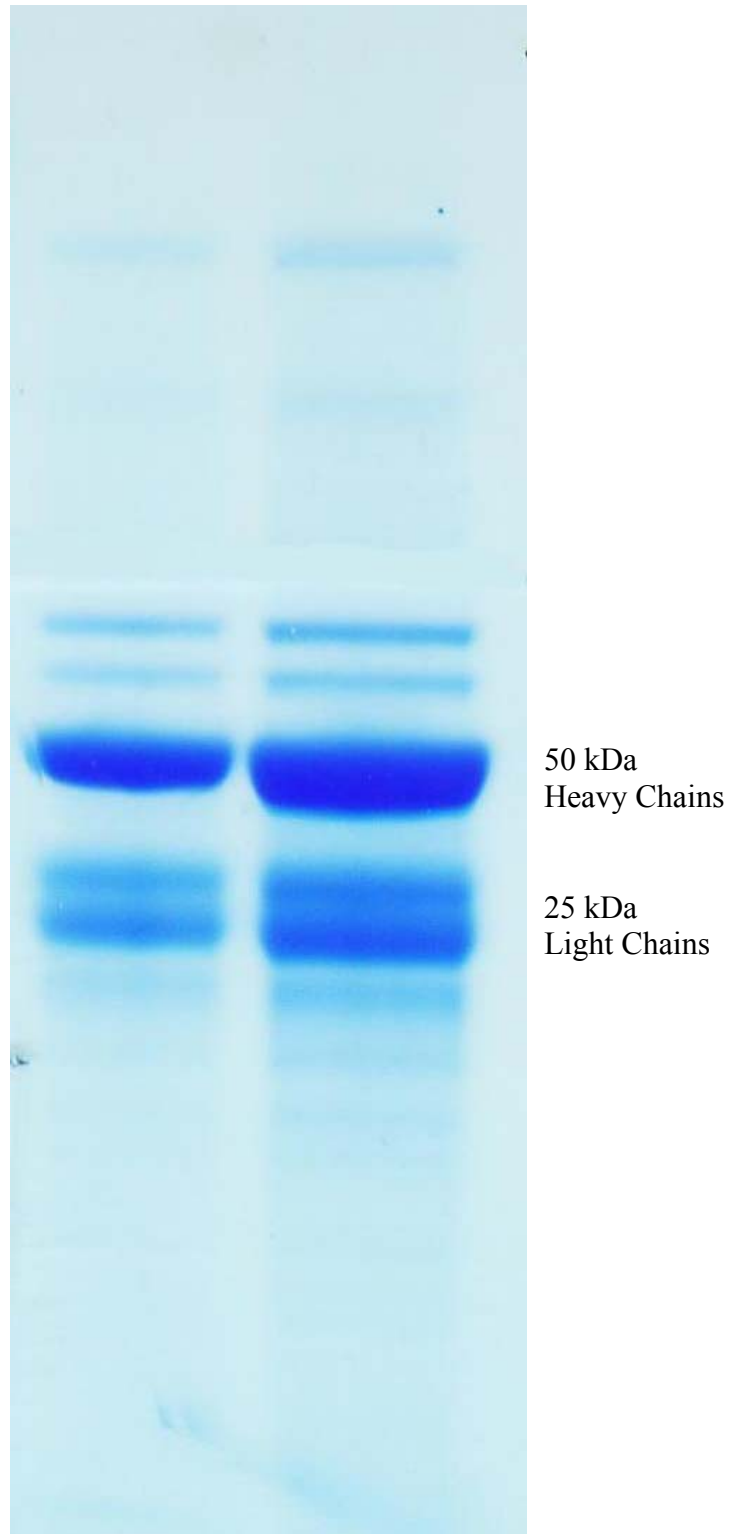


Figure 4: SDS-PAGE confirming the presence of the monoclonal antibody 25-D1.16. Left lane contains 5 μ l of sample; the right lane contains 10 μ l. The darker, thicker band is the immunoglobulin heavy chain; the two smaller bands are the two light chains (below the heavy chain).

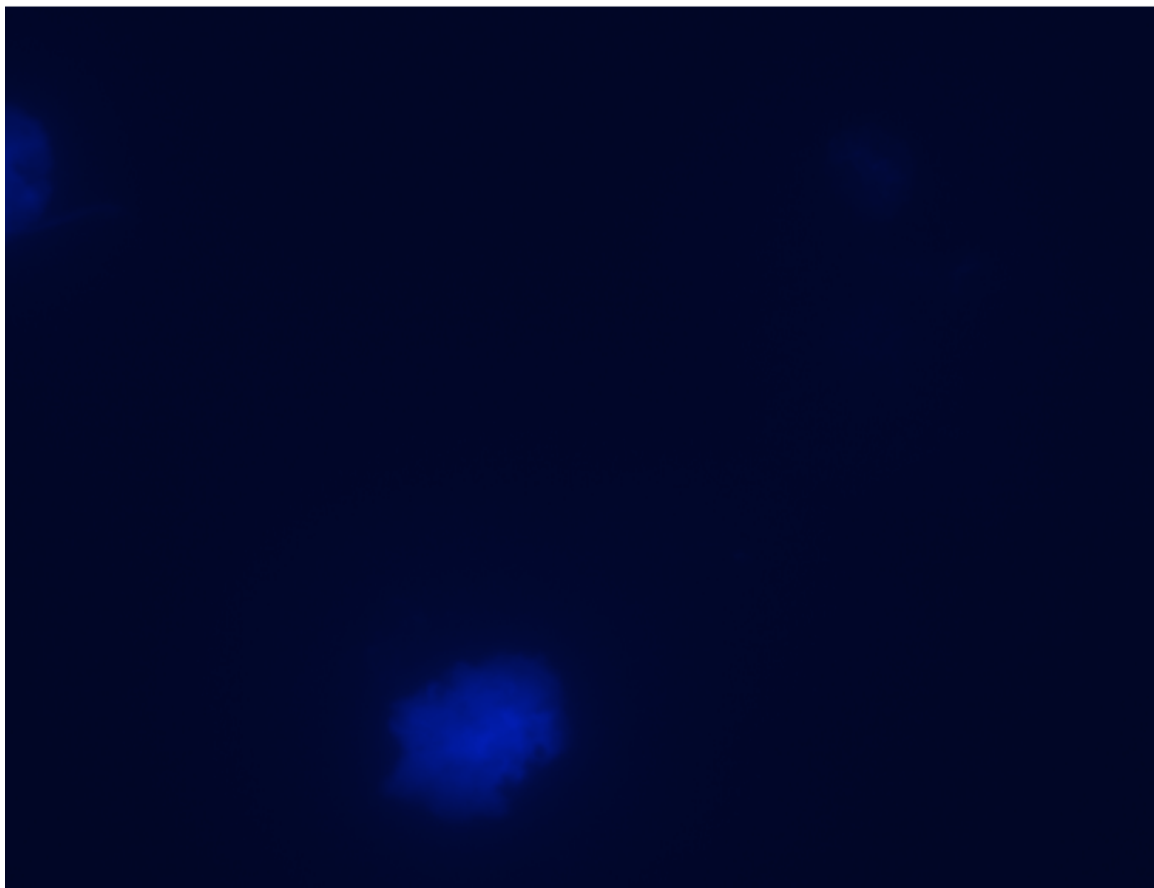


Figure 5: DAPI-stained APC with 0.15625 μM of TAT-mHER2 antigen (DAPI filter, magnification $\times 1000$).

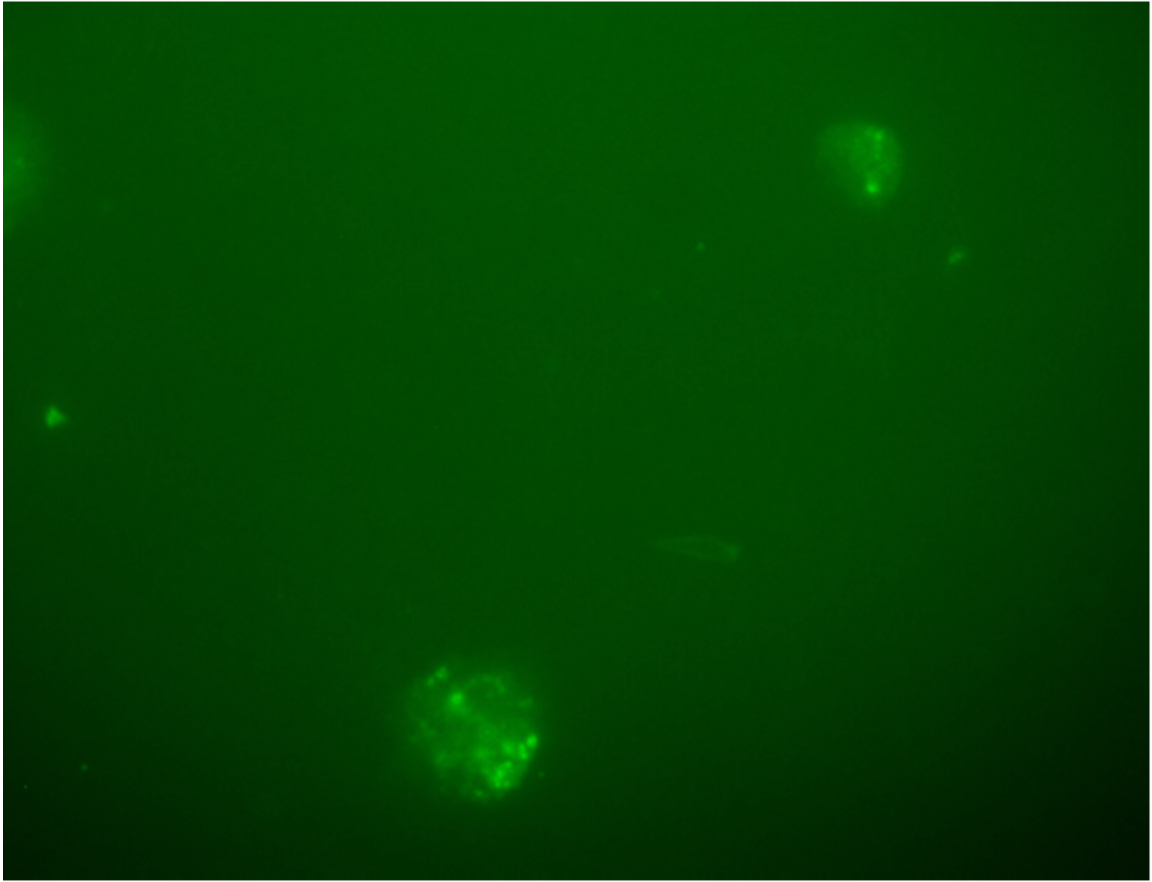


Figure 6: TAT-mHER2 (0.15625 μ M) expressed on APC cell surface bound to 25-D1.16 mAb which is bound to biotin-conjugated rat anti-mouse immunoglobulin and stained with SAV-FITC; binding sites are intensely bright points (FITC filter; magnification $\times 1000$). Note: the FITC-stained object in the upper-right corner is background interference.

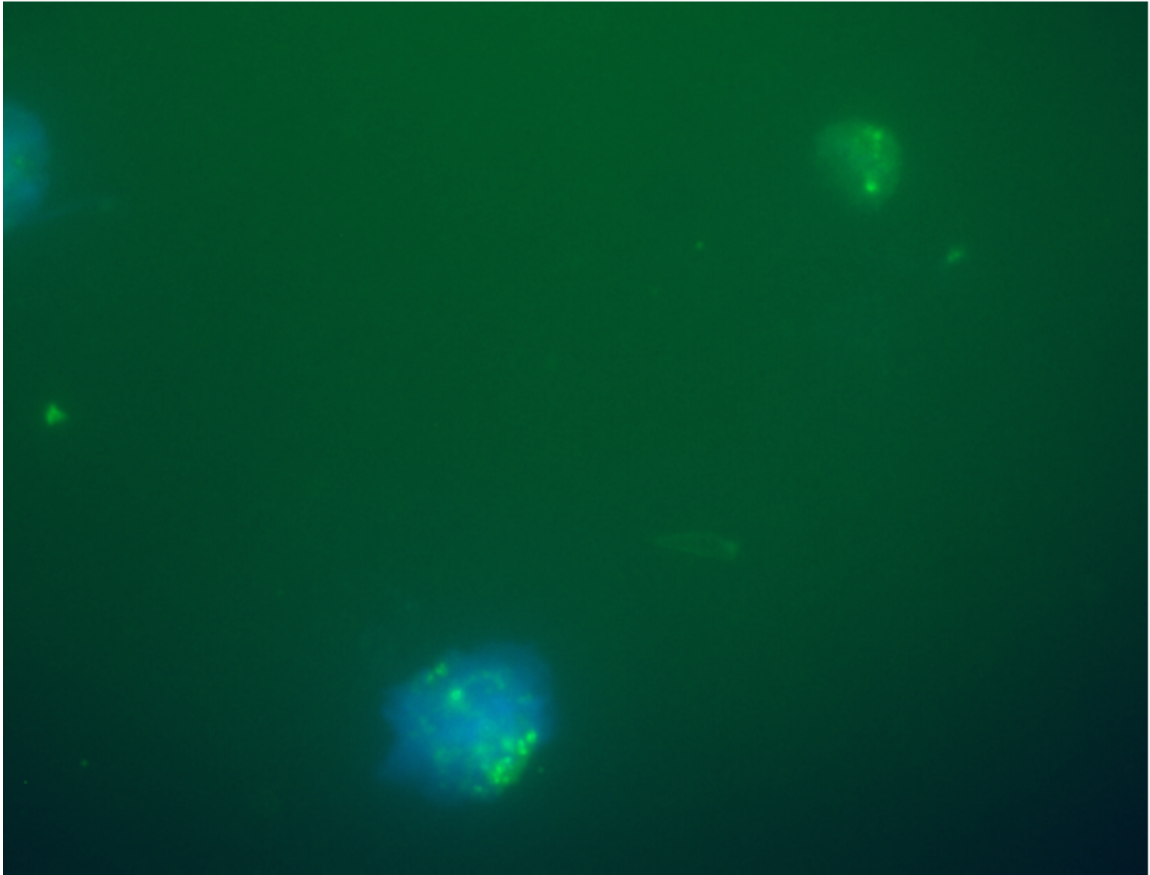


Figure 7: DAPI-stained APC and FITC-stained TAT-mHER2 (0.15625 μM) overlay (DAPI and FITC filters overlaid; magnification $\times 1000$). Note: the FITC-stained object in the upper-right corner is background interference.

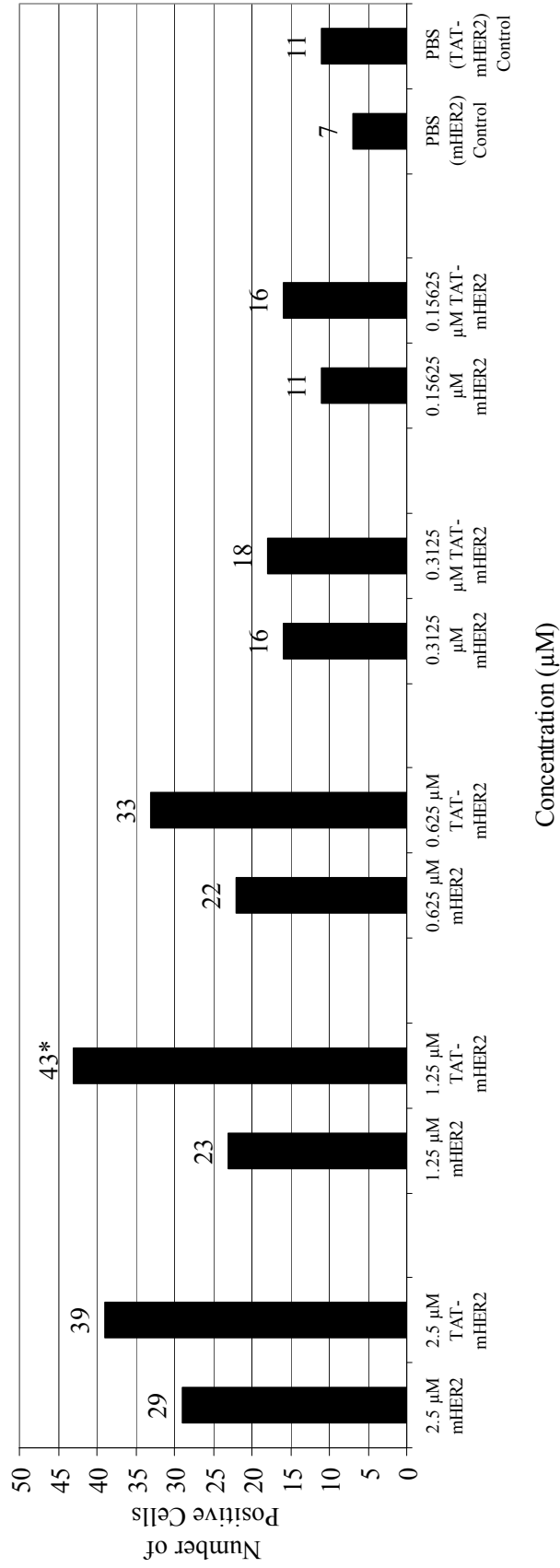


Figure 8: Number of cells positive for mHER2 and TAT-mHER2 from antigen concentrations of 2.5 μM to 0 μM. The asterisk denotes a statistically significant p-value.

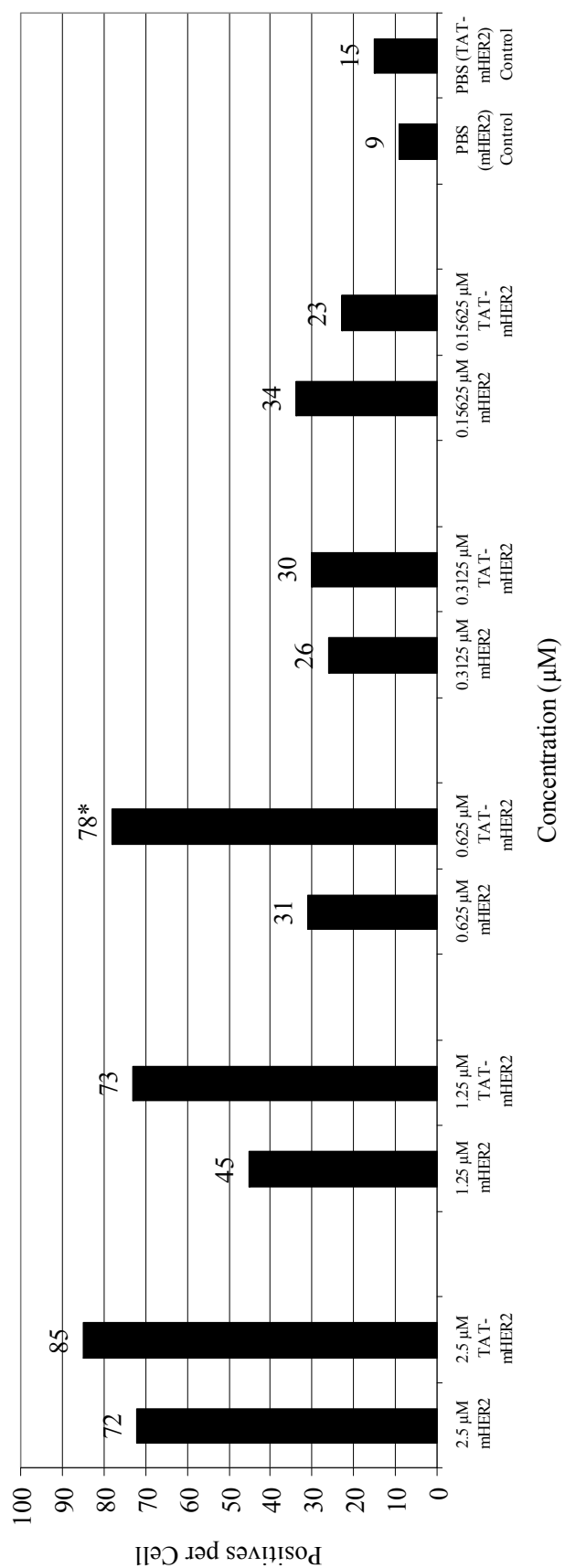


Figure 9: Number of positive signals per cell for mHER2 and TAT-mHER2 from antigen concentrations of 2.5 μ M to 0 μ M. The asterisk denotes a statistically significant p-value.

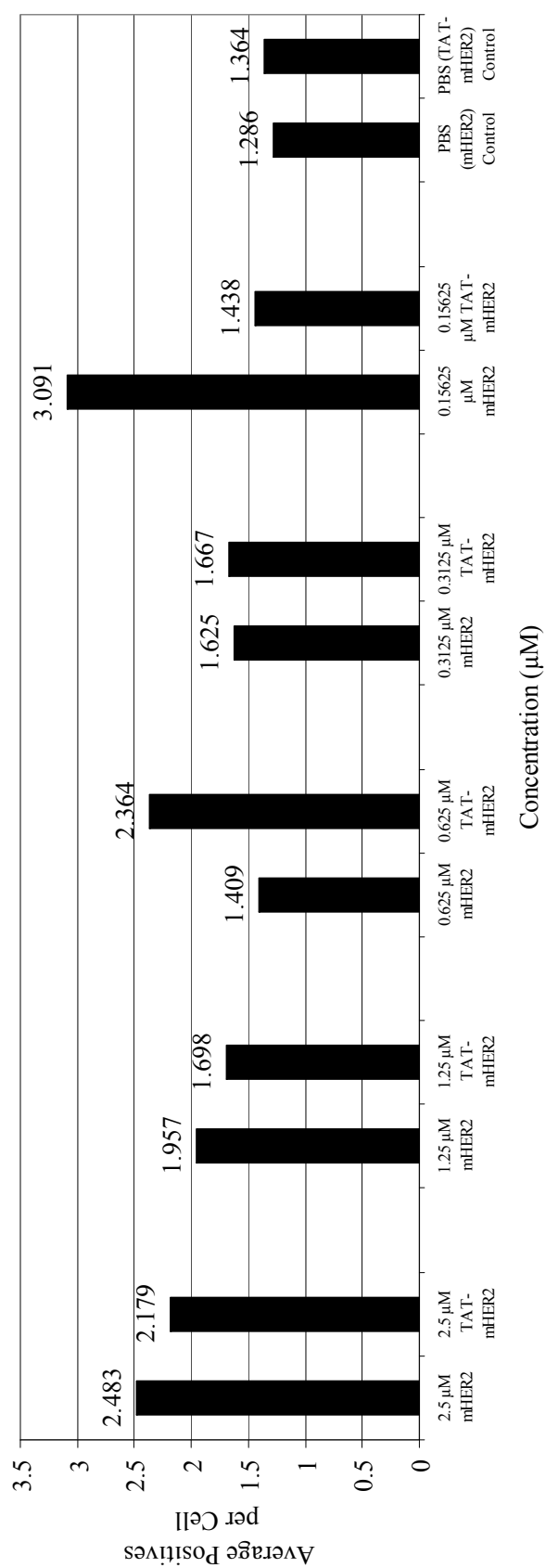


Figure 10: Average number of positive signals per cell for mHER2 and TAT-mHER2 from antigen concentrations of 2.5 μM to 0 μM.

APPENDIX B

APPLICATION TO USE VERTEBRATE ANIMALS




Northern
Michigan
University

Continuing Education & Sponsored Programs
1401 Presque Isle Avenue
Marquette, MI 49855-5325

May 25, 2007

TO: Robert J. Winn
Biology Department

FROM: Cynthia A. Prosen, Ph.D. 
Dean of Graduate Studies & Research

RE: **Application to use Vertebrate Animals**
Application # IACUC 060
Approval Period: April 11, 2007-April 30, 2010

The Institutional Animal Care and Use Committee approved your application for your project to use vertebrate animals in research entitled "Antigen Presentation by Spleenocytes and Dendritic Cells".

If you have any questions, please contact me.

kjm

cc: Biology Department